THE DEVELOPMENT OF CARRIERS AND ADJUVANTS FOR USE WITH PEPTIDES TO INDUCE MUCOSAL AND SYSTEMIC IMMUNITY AGAINST BIOLOGIC TOXINS

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13. ABSTRACT (Maximum 200 words)

Novel methods were used to enhance the immunogenicity of peptides of biologic toxins in order to elicit secretory and systemic anti-toxin antibodies. Studies were focused on model peptides of two classes of toxins: a) plant lectin toxins (Ricin), and b) bacterial toxins (staphylococcal enterotoxin B [SEB]). Since Ricin toxicity was shown to be abolished by neutralizing monoclonal antibodies to either the A chain - the polypeptide chain responsible for ribosomal inhibition or the B chain containing the galactose binding site which facilitates cell entry, sets of overlapping peptides spanning regions containing putative neutralizing epitopes on the two chains were synthesized. The minimal necessary specificity for the neutralizing monoclonal antibody to the A chain was defined as the 10 amino acid sequence, NQEDAEAITH representing a small

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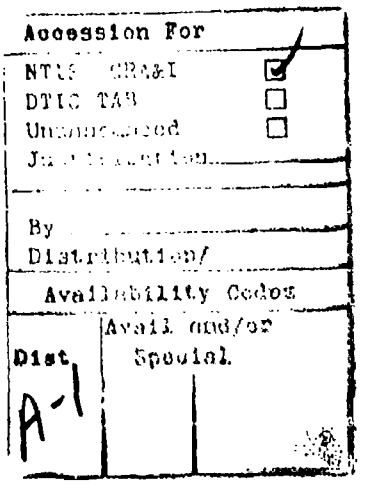
helical region projecting externally from the rim of the enzymatic cleft. Peptides were hydrophobically complexed to proteosomes through a cysteinyl-lauric acid anchor or covalently linked to KLH. Mice immunized with a conformation fixed cyclic peptide containing the neutralizing epitope on the Ricin A chain were shown resistant to Ricin toxicity at a limited dose range. Systemic and mucosal immunity in small animals was measured by examining the development of secretory antibodies (of all classes) in lung and gastrointestinal secretions as well as serum antibodies (especially IgA). This immunization system was used successfully for the enhancement of gastrointestinal and respiratory secretory and systemic immunity using oral and intranasal immunization protocols. The synergistic roles of associated oral adjuvants such as CTB and LPS was examined.



FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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1. INTRODUCTION

Protection against biologic toxins may necessitate the development of both gastrointestinal (GI) and respiratory secretory immunity as well as systemic immunity. In general, these toxins may be classified into three distinct groups: a) plant lectin toxins (e.g. Ricin) (1,2), b) large molecular weight bacterial toxins, (e.g. staphylococcal enterotoxin B [SEB]) (3), and c) small molecular weight peptide toxins (e.g. scorpion neurotoxin, NTP2) (4,5). In order to protect against such toxins, immunogens must be developed that are not themselves toxic when administered by either the parenteral or mucosal route. In the past, formalin treatment of toxins (toxoids) has been adequate for certain toxins (e.g. tetanus toxoid). Such methods, however, can result in either insufficient inactivation of the toxin (with concomitant host toxicity) or denaturation of the toxin (which renders it unable to induce antibodies that neutralize its activity) (1). Recently, advances have been made in the elucidation of the composition, structure and function of these toxins that have increased the feasibility of the development of protection using sub-unit peptide vaccines (6). Whereas the known structure-function relationships of each of these toxins varies considerably, and whereas each of the toxins presents individual problems in selecting the appropriate peptide epitope important for neutralization of toxicity, the basic question of how best to induce both mucosal (respiratory and GI) and systemic immunity is common to the development of any vaccine against these toxins.

1.1 SELECTION OF TOXINS

The toxins selected for this study were suggested by George H. Lowell, M.D. LTC MC - Medical Liaison Officer from the USAMRDC to Israel that worked as a Visiting Scientist in our laboratory at the Weizmann Institute of Science. They were selected according to the following considerations:

a) *Ricin*

1) The primary amino acid sequence has been determined. 2) The crystallographic structure has been analyzed and the three dimensional structure of both the A chain and the B chain of ricin has been elucidated. 3) A binding cleft with the putative active enzymatic site has been identified in the A chain allowing for the rational selection of peptide epitopes that may induce antibodies that directly interfere with its toxic activity. 4) The galactose binding sites of the B chain (i.e. the critical amino acids that are necessary for binding and incorporation of the toxin into cells) have been identified allowing for the rational selection of peptide epitopes that act to block cell penetration. 5) *In vitro* (cellular cytotoxicity as well as cell-free assays) and *in vivo* assays have been well described and can be readily and safely performed according to

established methods. 6) Formalin treatment to toxoid ricin does not sufficiently inactivate the toxin even after prolonged treatment. 7) USAMRDC investigators have expressed interest in using anti-ricin antibodies (Dr. Nick Johnson, Chief, Dept. of Nephrology, WRAIR).

b. *Seb (staphylococcal enterotoxin B)*

1) The primary amino acid structure is known. 2) A collaboration has been established with the Dept. of Chemical Immunology of the Weizmann Institute and Dr. Peter Gemski, Chief, Dept. of Molecular Pathology, WRAIR, to continue the investigation of the immunogenicity of SEB peptides. Peptides and lipopeptides ordered via this collaboration from Peninsula laboratories by the Dept. of Molecular Pathology, WRAIR, were made available to Dr. Lowell for immunogenicity studies; antibodies that were elicited were made available to Dr. Gemski for functional activity in assays testing for neutralization of the toxin.

1.2 PEPTIDE VACCINE DEVELOPMENT FOR ANTI-TOXIN IMMUNITY - PREVIOUS ACHIEVEMENTS IN OUR LABORATORY

The concept of peptide vaccines was first pioneered in this laboratory. Following our initial report that synthetic peptides could elicit anti-protein antibodies (7), it was predicted that molecular engineering would make it possible to design multivalent synthetic vaccines (8). During the past decade, we have applied the principles of peptide vaccine development to several biologic toxins and have successfully elicited anti-toxin immunity against diphtheria toxin (9), cholera toxin (10) and shiga toxin (11). The antibodies induced effectively neutralized the toxins both *in vitro* and *in vivo* (12). Furthermore, we showed that peptide vaccines could protect against the lethal effects of such biologic toxins (11). In these studies we have extensively explored several parameters that enhance the immune response to peptide antigens including a) the role of proteins, fatty acids or synthetic polypeptides to serve as peptide carriers (13) and b) the ability of synthetic adjuvants such as muramyl dipeptide (MDP) to enhance these responses (14). We have also examined several protocols to develop the preferred combination oral and parenteral immunization for the induction of anti-toxin secretory immunity (15).

1.3 THE USE OF PROTEOSOMES TO ENHANCE ANTI-TOXIN IMMUNITY

Proteosomes are preparations of meningococcal outer membrane proteins whose hydrophobic nature causes them to naturally form multimolecular, membranous vesicles (or vesicle fragments) (16-19). Like *E. coli* and salmonella outer membrane proteins which have been used as immunopotentiating agents (20, 21), they mitogenically activate proliferation and antibody secretion of both murine and human B cells (22-24). They are particularly relevant to vaccine development since these proteins have been safely given to thousands of persons in the development of meningococcal vaccines (25). For these safety,

physicochemical and immunologic reasons, they were selected to serve as both protein carrier and adjuvant in the development of peptide vaccines. Indeed, proteosomes were shown to be highly effective in enhancing the immunogenicity of trypanosome, malaria or streptococcal peptides even without added adjuvants (16-19). Anti-peptide antibodies induced by these proteosome-hydrophobic anchor vaccines recognized native or recombinant proteins or whole organisms (sporozoites). In these studies, immunogenicity was strongly influenced by a) the length and orientation of the peptide and the hydrophobic anchor, b)epitope repetition and c) cysteine dimerization or cyclization of the peptides. Proteosomes have also been used to enhance the immunogenicity of gangliosides, influenza hemagglutinin and, most recently, a recombinant malaria protein custom-engineered with a octapeptide cysteinyl-hydrophobic anchor. Furthermore, in preliminary experiments, intraduodenal administration of proteosomes complexed to shigella LPS enhanced secretory anti-LPS IgA (26).

1.4 OBJECTIVES

The two major objectives of these studies are:

1. Identification of peptides that induce Ricin-neutralizing antibodies on the A and B chains of the protein.
2. Development of immunopotentiating methodology which will be designed to elicit secretory and systemic antibodies to peptides of Ricin and SEB.

1.5 HYPOTHESIS

Concerning the identification of Ricin-neutralizing antibodies, we hypothesize that the critical peptide epitopes that will elicit antibodies that neutralize Ricin will be identified based on the known structure of the A and B chains of Ricin. This is possible since the binding cleft of enzymatic activity has been visualized by crystallographic studies and since the galactose binding sites of the B chain of Ricin has similarly been shown. Furthermore, these sites correlate with studies showing that altering certain amino acids, modifies toxic activity.

Concerning the immunopotentiation of secretory and systemic immunity, we hypothesize that by covalently and/or hydrophobically complexing toxin peptides from Ricin and SEB to several different carriers and adjuvants and by comparing combinations of oral and parenteral immunization protocols, secretory and systemic immunity will be elicited against each of these toxins. Due to the varied nature of the peptides to be used, the optimal immunopotentiating regimen or carrier-adjuvant system may not be identical for each toxin. The carriers and adjuvants to be used will include natural proteins (proteosomes or other proteins). We further hypothesize that certain constructs will induce antibodies

that neutralize the toxin and that these studies will lead to the development of anti-toxin vaccines.

1.6 PEPTIDES INCLUDED IN THE STUDY

1.6.1. Ricin A Chain

Based on the published primary structure of ricin (27,28) and on the three dimensional structure as determined by x-ray crystallography at 2.8 Ångstrom resolution (29), a series of overlapping peptides spanning the putative cleft of enzymatic active site (Residues E177, R180, N209 and W211 (1) were synthesized. These peptides, which cover a total of 130 amino acids from a domain dominated by helical structures, are listed below (single letter amino acid code):

RA (91-116): Y F F H P D N Q E D A E A I T H L F T D V Q N R Y T
RA (107-132): L F T D V Q N R Y T F A F G G N Y D R L E Q L A G N
RA (122-149): Y D R L E Q L A G N L R E N I E L G N G P L E E A I S
RA (144-170): L E E A I S A L Y Y Y S T G G T Q L P T L A R S F I I
RA (161-186): L P T L A R S F I I C I Q M I S E A A R F Q Y I E
RA (171-196): C I Q M I S E A A R F Q Y I E G E M R T R I R Y N R
RA (199-221): A P D P S V I T L E N S W G R L S T A I Q E
RA (205-213): T L E N S W G R L

At a later stage of this study, when peptide RA(91-116) was shown to bind to a monoclonal antibody that neutralizes the toxin, (Figure 1), additional shorter peptides were synthesized to define the minimal sequence of the neutralizing epitope (Figure 2).

1.6.2 . Ricin B Chain

Ricin sugar binding domains were described as shallow pockets with their top formed by a side chain of an aromatic residue (Trp 37 and Tyr 248 for the two sites respectively) and bottom by a peptide backbone of three residue kinks (Arg 24 Asp 25 Gly 26 and Arg 236 Ala 237 Ser 238, respectively). Conserved aspartic acid seems crucial in the two sites (Asp 22 and 234, respectively). Hydrogen bonding of the aspartic residues to glutamic residues (Glu 47 and 256, respectively) stabilizes the aspartic acids orientation. Hydrogen bonds are formed between Asn 46 and 255 and bound galactose residues.

Peptides consisting of sequences containing the relevant amino acids were synthesized including control peptides from the two putative sites lacking the relevant amino acids.

RB(20-47): C V **D** V **R** **D** **G** R F H N G N A I Q L **W** P C K S N T D A **N** **Q**
RB(20-28): C V **D** V **R** **D** **G** R F
RB(28-36): F H N G N A I Q L
RB(33-47): A I Q L **W** P C K S N T D A **N** **Q**
RB(230-257): G L V L **D** V **R** **A** **S** D P S L K Q I I L Y P L H G D P **N** **Q** I
RB(232-239): V L **D** V **R** **A** S D
RB(239-247): D P S L K Q I I L
RB(244-262): Q I I L **Y** P L H G D P **N** **Q** I W L P L F
RB(247-256): L Y P L H G D P N Q

1.6.3. SEB

The following peptides were available (with a lauryl group linked to the amino terminus and plain) by collaborative arrangement with Dr. Peter Gemski, Chief, Dept. of Molecular Pathology, WRAIR.

1. 1-30 : E S Q P D P K P D E L H K S S K F T G L M E N M K V L Y D D
2. 21-50 : M E N M K V L Y D D N H V S A I N V K S I D Q F L Y F D L I
3. 41-70 : I D Q F L Y F D L I Y S I K D T K L G N Y D N V R V E F K N
4. 61-92: Y D N V R V E F K N K D L A D K Y K D K Y V D V F G A N Y Y Y Q
5. 83-113: D V F G A N Y Y Y Q **C** Y F S K K T N D I N S H Q T D K R K T **C**
6. 93-112: C Y F S K K T N D I N S H Q T D K R K T
7. 101-130: D I N S H Q T D K R K T **C** M Y G G V T E H N G N Q L D K Y R
8. 113-144: C M Y G G V T E H N G N Q L D K Y R S I T V R V F E D G K N L L
9. 130-160: R S I T V R V F E D G K N L L S F D V Q T N K K K V T A Q E L
10.151-180: N K K K V T A Q E L D Y L T R H Y L V K N K K L Y E F N N S
11.171-200: N K K L Y E F N N S P Y E T G Y I K F I E N E N S F W Y D M
12.191-220: E N E N S F W Y D M M P A P G D K F D Q S K Y L M M Y N D N
13..210-239: Q S K Y L M M Y N D N K M V D S K D V K I E V Y L T T K K K

2. EXPERIMENTAL METHODS

2.1 PEPTIDES

All peptides were synthesized at the Weizmann Institute peptide synthesis service on applied biosystems model 430A peptide synthesizer using PAM resins and suitably protected t-Boc amino acids.

The peptides were designed to have in addition to the native form, a version with a terminal cysteine residue for covalent linkage to KLH carrier and another with terminal cysteinyl lauryl group to serve as hydrophobic anchor that enables

the lipopeptides to complex with proteosomes (16-19). The peptides were partially purified by gel filtration on Sephadex G-25 column (Pharmacia, Uppsala, Sweden) using either different concentrations of glacial acetic acid or 1% solution of Empigen BB buffered to 8.5 with Tris EDTA and NaCl (TEEN) as eluents according to the peptides solubility. Some of the peptides were checked for purity on reversed phase HPLC and all had the expected amino acid analysis.

2.2 PEPTIDE CONJUGATION TO CARRIERS AND ADJUVANTS:

The ability of several carriers and adjuvants to enhance toxin peptide immunogenicity was compared. Peptides were hydrophobically complexed to proteosomes by methods previously described in detail, namely, via the hydrophobic foot consisting of a cysteinyl-lauric acid, added to the carboxy terminus of each peptide, which was then hydrophobically complexed to proteosomes by the dialysis method as described (16). In addition, covalent complexes were made by linking peptides with carboxy terminal cysteine to KLH via MBS.

Outer membrane proteins were extracted from group B serotype 2b meningococci (Proteosomes) as described (25). Hydrophobic binding of the lipopeptides to the proteosomes was done by resuspending them at 4:1 ratio (W/W) in TEEN followed by exhaustive dialysis across a 1,000 molecular weight cutoff membrane against PBS for 8-10 days. Covalent conjugation of NH2 terminal cysteine peptides to KLH was done by using MBS (m-maleimido benzoyl hydroxyl sulfosuccinimnide ester) (Pierce, IL.USA) as cross linker of terminal cysteine SH groups of the peptides with the amino groups of lysins on the KLH (Pierce .IL, USA) as described (30).

2.3 IMMUNIZATION

Mice were immunized with the immunogens parenterally (in saline or with complete Freund's adjuvant), orally, or intra-nasally in a variety of regimens to determine the optimal protocol to induce both mucosal and systemic immunity (15,31).

2.3.1 Parenteral Immunization

Groups of outbred CD1 mice were immunized intraperitoneally without adjuvant with vaccines containing 50ug of peptides as proteosomes complexes and boosted with the same vaccine 3 and 7 weeks later. Sera were collected 1-2 weeks after immunization. KLH vaccines were given at the same dose and schedule into the footpads with CFA as adjuvant for prime injections and IFA for boosts.

2.3.2 Intranasal Immunization

Groups of Balb/C mice were immunized intranasally either by one or several 50ul applications of proteosome based vaccines. One week after the last application animals were anesthetized with ether and bronchial lavage fluids (0.5% BSA in PBS as washing solution) and blood were collected. In some experiments lavage fluids were collected also from the guts in the presence of protease inhibitors in the lavage fluids. An alternative protocol of intransal immunization, using 2 series of administration, 3 weeks apart, of 3 bi-daily immunizations, was used to elicit higher level of secretory immunity.

2.4 MONOCLONAL ANTIBODIES

Ricin neutralizing monoclonal antibodies prepared against the A polypeptide chain were obtained from Cetus Corp. Neutralizing monoclonal antibodies against the B chain of ricin were prepared for us by the Biological Services - Antibody unit of the Weizmann Institute.

2.5 ELISA FOR MEASURING ANTIBODY TITER.

Sera were assayed for anti homologous peptides and protein specificities by ELISA. The 96 well polystyrene plates were coated with 10ug/ml protein in carbonate buffer pH 9.6 for 1 h at 37C or with 2ug/ml of peptides and allowed to dry overnight at R.T. The unbound antigen was washed away and the plates were incubated for an hour with 1% filler casein for 1 h at 37C. The plates were washed with 1% Tween-PBS, and the samples diluted in filler solution were added. After overnight incubation at RT the plates were washed and Horseradish peroxidase labelled goat anti mouse IgG was added. after 24 h at RT the plates were washed and developed with 2,2"-azidodi-(3-ethylbezthiazoline sulfonic acid) diammonium salt (ATBS, Sigma St. Louis, MO. USA) in citrate buffer pH 4.0 in the presence of 0.003% hydrogen peroxidase. Absorbance was recorded at 405 using a microplate reader (Titertek Multiskan,MC).Test controls ensuring specificity included the use of heterologous peptides as detecting antigens and sera of animals immunized with either saline, heterologous peptides or carrier alone. Results are presented as a mean of triplicates.

2.6 SOLUTION PHASE BINDING ASSAYS

The assay was carried out as in the preceding paragraph except the antibody samples were mixed with increasing concentrations of antigens in solution (either peptides or Ricin proteins) before incubation. Results were calculated as percentage of binding levels in the presence of soluble antigen over those obtained in its absence.Results are presented as a mean of triplicates.

2.7 NEUTRALIZATION OF RICIN TOXCITY

Toxin neutralization was evaluated both *in vitro* and *in vivo.*

2.7.1 *In vitro* Studies

Toxin neutralizing antibodies were assessed by their capacity to inhibit toxicity. Ricin toxicity assays were performed in 96 microtiter plates. Freshly washed NSO myeloma cells at 10^5/well in 50ul DMEM supplemented with 10% FCS were incubated overnight at 37C with different concentrations of Ricin (Sigma, St. Louis, Mo.) preincubated overnight at 4C with the antibodies to be tested in 50ul of serum free medium. At the end of the incubation period MTT (Sigma, St. Louis, Mo.) reaction for living cells was carried out as described (32). Stock solution of 5mg/ml MTT in PBS (30 ul) was added to each well and plates were incubated at 37C for 4 h. Acid-isopropanol solution (15ul of 0.05N HCl in isopropanol) was added to the wells and mixed thoroughly to dissolve the dark crystals. The plates were read using a microplate reader (Titertek Multiscan) at 570 nm as test wave length and 630 nm as reference.

2.7.2 *In vivo* Studies

In vivo toxin neutralization was assessed by active and passive immunization of mice with the peptide vaccines. Antisera to SEB peptides were sent to Dr. P. Gemski at WRAIR for functional assays and for comparison with KLH-covalently coupled peptide vaccines, whereas antisera against Ricin peptides were evaluated in our laboratory.

2.7.2.1 *Neutralization by Passive Immunization*

Ricin at different doses was incubated for 1 h at 4C with the antiserum or antibody to be tested for neutralizing activity in 0.15% BSA/PBS. Groups of CD1 outbred mice were injected i.p. with 300ul Ricin solution. Animals death was monitored.

2.7.2.2 *Neutralization by Active Immunization*

Groups of CD1 outbred mice were immunized with the different vaccines as described in 2.3.1 and then injected i.p. with different doses of Ricin in 300 ul 0.15% BSA/PBS. Animals death was monitored.

3. RESULTS

3.1 RICIN PEPTIDE VACCINES

To elicit neutralizing immunity against Ricin using analogous synthetic peptides, two approaches were pursued. The first approach utilized sequences from the putative active site of the enzymatic activity (Ricin A peptides) and the second from the part of the B chain containing the major galactose binding site which facilitate cell entry (Ricin B peptides).

3.1.1. Ricin A Chain

3.1.1.1 *Determination of the Epitope recognized By A Neutralizing Monoclonal Antibody.*

Based on the published primary structure of Ricin (27,28) and on its 3 dimensional structure as determined by x-ray crystallography at 2.8A resolution, a series of overlapping peptides spanning the putative enzymatic active site were synthesized. These peptides covered a total of 130 amino acids from a domain dominated by helical structure.

The identification of the peptide representing the epitope for a neutralizing monoclonal antibody to Ricin was accomplished using an ELISA inhibition assay measuring the ability of the soluble peptides to inhibit the binding of the monoclonal antibody to Ricin, where Ricin A chain was used as the solid phase antigen bound to ELISA plates (FIGURE 1). As shown, from the panel of peptides used, spanning residues 91-221 of Ricin A chain sequence, only RA91-117 was found to prevent the neutralizing mAb from binding to Ricin A protein (50% inhibition with about 16ug/ml [6.6x 10^{-6}M] peptide. An overlapping peptide containing the sequence 107-132 was not inhibitory indicating that the the recognized area is within amino acids 91-110. This was confirmed and further defined by synthesizing and testing additional peptides as shown in FIGURE 2. First, three 10-mers, 91-100, 96-105 and 101-110, were shown to be ineffective (inhibition <24%).The 15-mer, 96-110, how exhibited 81% inhibition. Therefore, to demonstrate the minimal necessary specificity, a series of shorter peptides was synthesized by successively decreasing 96-110 from either the amino or carboxyl termini. In this manner it was shown that aspargine 97 was critical for binding, since its elimination markedly decreased mAb recognition; 97-110 gave 78% inhibition whereas 98-110 inhibited only to the extent of 37%. On the carboxyl side, histidine 100 was determined to be the last amino acid necessary for significant binding to the neutralizing mAb since 96-108, 96-107 and 96-106 inhibited 91, 87 and 85%, respectively, whereas 96-105 inhibited only 24%. Thus the epitope recognized by the neutralizing mAb was localized to 10 amino acids 97-106. This sequence represents a small helical region externally projecting from a domain dominated by beta-stranded sheets.

3.1.1.2. *Immunogenicity and Ricin Specificity of Peptide Vaccines*

Vaccines were prepared with each of the synthesized peptides and not only to those recognized by the neutralizing mAb, since the specificity of this mAb represents only one neutralizing epitope and it was considered likely that other neutralizing epitopes may be represented by peptides in the vicinity of that recognized by the mAb. Sera were tested for antibodies recognizing homologous peptides and Ricin A protein. After three immunization, serum IgG recognizing both homologous peptides and Ricin A protein with titers of at least 1:1600 were induced to each of the selected Ricin A areas with the exception of areas 5 and 8 where lower (1:400) antipeptide titers and no detectable anti-protein antibodies were found (TABLE Ia). The Proteosome/NS vaccines were generally more effective than KLH/CFA system (see especially peptide 190-215) in inducing both anti-peptide and anti-Ricin A protein antibodies. Included among the high tittered sera were regions 171-196 and 161-186 which contain amino acids glutamine 177 and arginine 180. These amino acids, considered important for enzymatic activity, are located within the putative enzymatic cleft indicating that antibodies were successfully made that recognize areas within the cleft as well as either side of it. Although anti-Ricin A protein IgG was induced by the 15-mer and 20-mer peptides recognized by the neutralizing mAb (91-110 and 96-110), the shorter peptides in this area that more exactly defined the mAb's specificity were poorly immunogenic (e.g. 96-106, 96-107 etc.) (TABLE Ib).

3.1.1.3 *Comparison of Antigenic Repertoire of Polyclonal Antibodies Induced by Peptide 91-110 and 96-110 with That of the Neutralizing MAB*

The immune repertoire of antisera induced by peptides 91-110 and 96-110 (using proteosomes or KLH, respectively) was compared to that of the neutralizing mAb, by testing the ability of the panel of related peptides to inhibit binding of these antibodies to Ricin A. As shown in FIGURE 3, binding of both polyclonal antisera was almost completely inhibited by the 20-mer peptide 91-110. More specifically, greater than 90% inhibition of binding of these polyclonal antibodies were obtained by the locus defined by peptides 98-110, 99-110 and 100-110. In contrast, these peptides inhibit binding of the Ricin neutralizing mAb only to the extent of 18-26%, whereas maximum inhibition of this mAb was found using peptides 96-107 and 96-108. This implies that the dominant fine specificity of antibodies induced by vaccines using peptides 96-110 or 91-110 is preferentially directed toward recognition of an epitope that is shifted toward the carboxyl terminus of the protein (100-110) and that is not recognized by the mAb. Thus, although the mAb preferentially recognizes an epitope shifted only 3-4 amino acids towards the amino terminus of the protein (defined by 97-106), this small difference may account for differences in the ability of the polyclonal and monoclonal antibodies to neutralize Ricin. It is noteworthy that in repeated experiments, high amounts of the most fitting peptide (96-108) were needed to inhibit binding of the mAb (for 91% and 77% inhibition 50 and 16ug/ml

respectively) whereas the peptides could virtually totally abolish the binding of the anti-peptide polyclonal sera to Ricin protein at lower concentrations. This suggests that even those peptides found to have maximum specificity for the mAb (96-107 and 96-108) still did not assume the optimal configuration of the protein neutralizing site recognized by the mAb. This conclusion is supported by the results of immunization with vaccines containing the shorter peptides 96-107 and 96-108 which induced antibodies that very poorly recognized Ricin A protein (TABLE I b).

3.1.1.4 *Effect of Soluble Ricin A on Binding of Selected Polyclonal Antisera or the Neutralizing MAb to Solid Phase Ricin A in ELISA*

In order to clarify the basis for the differences between the abilities of the monoclonal antibody and the anti-peptide antisera to neutralize Ricin, since we found differences in fine specificity between the neutralizing mAb and the polyclonal antisera tested, we decided to investigate the ability of soluble Ricin to inhibit binding of these antisera to Ricin A protein in the solid phase as used in the ELISA. Indeed, we found that although polyclonal antibodies directed against these peptides bind to solid phase Ricin A protein in the ELISA (TABLE I), and although peptides in solution will inhibit binding of such antisera to solid phase Ricin (FIGURE 3), Ricin A protein in solution could not inhibit anti-peptide antisera from binding to Ricin A on the plate even when concentrations of 100ug/ml of soluble Ricin A protein were used (FIGURE 4). In marked contrast, soluble Ricin A inhibited binding of the neutralizing mAb to a level of 50% at a concentration of 0.1ug/ml. Interestingly, FIGURE 4 also demonstrate clearly that it is possible to inhibit anti-peptide antisera with Ricin A protein as shown by peptides 107-132 and 144-170. Although 100ug/ml were needed for 50-60% inhibition, this is not unreasonable since an equimolar amount of a 20-mer peptide would necessitate a 10-fold more of the 215 amino acid Ricin A protein and since 16ug/ml of such peptides were required for inhibition as shown in FIGURE 4. These data indicate that the dominant conformations in solution assumed by the Ricin A peptides described up to here a) do not mimic the conformation of soluble Ricin and b) induce antibodies with specificities for the peptides that mimic solid phase Ricin A protein as fixed to the ELISA plate but do not mimic the native conformation assumed by the protein in its soluble form. The above results and unsuccessful attempts to neutralize Ricin toxicity *in vitro* and *in vivo* (as described in the next paragraph) led us to think that the knowledge about the exact sequence of the neutralizing epitope should be combined with that about its configuration in the native protein to design a vaccine that conserve the natural conformation of the epitope. Such second generation vaccines are described in paragraph 3.1.1.6.

3.1.1.5. *Effect of the MAb to Ricin A Chain and Polyclonal Antisera Induced by Peptide Vaccines on Ricin In Vitro and In Vivo*

Neutralization of Ricin toxicity by antisera was assayed *in vitro* by preincubation of pre-determined concentrations of Ricin with sera prior to and during testing of the effect of Ricin on cell viability. As shown in FIGURE 5A, 0.7 ng/ml Ricin induced 50% cell death as monitored by the tetrazolium salt MTT reaction. Incubation of Ricin with concentrations of the neutralizing mAb as low as 0.4ng/ml ($2.6\ 10^{-9}$M) virtually abolished toxicity. In contrast, none of the antisera (used as serum dilution of 1:100) induced by either the proteosomes or KLH vaccines containing any of the peptides listed in TABLE I had any effect on the *in vitro* toxicity (examples using the 15-mer, 20-mer and 26-mer which were recognized by the neutralizing mAb are shown in FIGURE 5B).

In vivo neutralization of Ricin was measured by pre-incubation of Ricin with antisera or mAb before i.p injection of the toxin to mice (TABLE II). A dose of 250 ng Ricin killed all injected mice. Preincubation of the Ricin with 30 ug of the anti-Ricin A mAb (but not with a mAb of irrelevant specificity) completely abolished toxicity. When anti-peptide sera or ammonium sulfate IgG fractions of such antisera (induced by either proteosomes or KLH vaccines) were used, however, although the sera had reciprocal ELISA titers against Ricin A protein of 3200-6400, no effect on Ricin toxicity could be detected. Vaccinated animals from which antisera were retrieved were not resistant to 330 ng of Ricin given i.p.

3.1.1.6. *Second Generation Peptide Vaccines - Conformation Fixed Peptides*

3.1.1.6.1. *Peptides design*

Computer analysis of the projected conformation of Ricin in the region recognized by the neutralizing anti Ricin A chain antibody suggested the use of a peptide RA90-110 as immunogen that, by appropriate reduction and oxidation of two cysteines designed at both peptide termini, will undergo cyclization and be fixed as to mimic the natural conformation of the epitope in the native protein. Lauric acid added to the carboxyl terminus of the peptide was used to complex it to proteosomes. To improve the presentation of the cyclic peptide on proteosomes, RA90-110 was synthesized with extension of ala-ala-ala inserted between the cysteine and the lauric acid at the carboxyl terminus to provide the peptide with more flexibility necessary for equilibrium at the desired native conformation. The peptide was reduced with DTT and oxidized by air in a dilute solution.

3.1.1.6.2. *Parenteral immunization*

Groups of mice were immunized with vaccines made by the conjugation of the two cyclic peptides with proteosomes. Tertiary responses were measured by

ELISA against Ricin A protein; titers of 400 and 800 (O.D.>0.5) were obtained, respectively, for the cyclic RA90-110 and the one with the 3 Alanine extension (RA-Ala90-110).

3.1.1.6.3. *Inhibition of anti-RA90-110 binding to Ricin A as antigen in ELISA by soluble Ricin A*

To check if fixation of the peptide conformation in a form resembling its 3 dimensional structure in the protein (analysis based on x ray crystallography data) renders antisera against it more reactive with native Ricin, we studied the effect of increasing amounts of soluble Ricin A on the binding of anti cyclic RA90-110 and RA-Ala90-110 to ricin A protein in the solid phase as coating antigen in ELISA (FIGURE 6). Under the assay conditions 50ug/ml of added Ricin A inhibited antisera to the cyclic forms of RA-Ala90-110 and RA91-110 by 68% and 46% respectively while antisera to the linear forms RA91-117 and RA96-110 were inhibited only by 27% and 20% respectively. These data indicate that the cyclic forms of the peptides and especially the one with the Ala-Ala-Ala foot were able to induce antibodies recognizing better the soluble native Ricin than the linear RA91-117 and RA96-110.

3.1.1.6.4 *Neutralization of Ricin toxivity in vivo*

Antisera to the described vaccine failed to neutralize Ricin toxicity by *passive immunization*, when mixed together before injection to animals (TABLE III). In the same experiment mAb to Ricin B (shown to neutralize Ricin toxicity *in vitro*) neutralized Ricin toxicity in a narrow dose range. However, on *active immunization*, animals immunized with the cyclic forms of RA90-110 and RA-Ala90-110 were shown to be resistant to Ricin toxicity, albeit in a narrow dose range (TABLE IV).

The two last experiments emphasize the importance of the ratio between the dose of Ricin and the amount of antibodies for the neutralization of the toxin. It is possible that failure to abolish Ricin toxicity in passive immunization experiments is due to insufficient amount of neutralizing antibodies (20ul of antisera per mouse as dictated by their availability) compared to the whole animal blood volume of vaccinated animals in active immunization experiments.

3.1.2. Ricin B chain

3.1.2.1 *Peptide Vaccines*

Data from 8 vaccines (7 hydrophobically-linked proteosome-lipopeptide vaccines (in saline) and 1 covalently-linked KLH-peptide vaccine (emulsified in Complete Freund's Adjuvant [CFA]) representing peptides from the two homologous galactose binding site on the Ricin B chain were analyzed (Table Va). Since two Ricin B peptides 230-257 and 244-262 from the 2nd binding site induced

antibodies that recognized the homologous peptides but did not recognize native Ricin B protein, five other peptides were synthesized, purified and vaccines were made from the first putative site (RB20-28,RB20-47) and the second (RB232-239,RB247-256).As with former peptides these vaccines induced anti peptide but not anti Ricin B protein antibodies. Peptide 230-257 was complexed to 5 different preparations of proteosomes to determine if a change in the presentation of this peptide induced IgG that recognize the protein (TABLE Vb). A new lot of meningococcal proteosomes made in Israel (MgC-I), and 3 proteosome preparations made by Dr. Mylan Blake at the Rockefeller Univ., N.Y.: MgC-R, and 2 gonococcal proteosomes: GC-Std-R, and GC-X-R (lacking class 3 protein). Significantly, the standard MgC proteosome induced the highest anti-peptide titers whereas the others induced better anti-protein titers, especially MgC-I, which elicited the poorest anti-peptide titers (Table V).Hence two new Ricin B peptide vaccines were made with the GC-X-R and MGC-I linked to 244-262 peptide which induced anti Ricin B titers of 25,000 (o.d.>0.5) with anti-peptide titers of 6,400 indicating the efficacy of this approach. Nevertheless, none of these vaccines induced detectable neutralizing antibodies in an *in vitro* assay using native Ricin; nor did the peptides inhibit the neutralization of Ricin B polyclonal sera.

3.1.3 Monoclonal Antibodies (MAb) Production

MAb to both A and B chains of Ricin were used to identify neutralizing epitopes of the toxin.

3.1.3.1. *Rlicin A Chain*

Sera of mice immunized with Ricin A chain showed a) high titered polyclonal anti-Ricin A antibodies by ELISA and western blots and b) low-titered activity in an *in vitro* assay developed to test for neutralization of native Ricin. Spleens of these animals were used for fusion and 40 mAbs were identified that recognized Ricin A. Although it was initially found that many of these antibodies had neutralizing activity as well, it was subsequently shown that the this was due to a factor in the horse serum used to grow the cells. When regrown in fetal calf serum none of the 40 MABs that recognized Ricin A chain in ELISA neutralized Ricin toxicity.

3.1.3.2 *Ricin B Chain*

Polyclonal antisera from mice immunized with Ricin B chain neutralized Ricin at a titer of 1;9000 after 2 boosts. From the hundreds of mAbs that bound to Ricin B chain in an ELISA, when the two hybridomas selected for sub-cloning were expanded and grown to ascites fluid, one clone was found to be superior in neutralizing toxicity *in vitro* as well as *in vivo*, when injected to animals together with the toxin neutralized its toxicity (TABLE III).

3.2. *SEB*

Proteosome vaccines made from 12 lauryl-cysteine peptides were tested for induction of binding antibodies to the native SEB protein and to homologous and overlapping peptides in an ELISA. Sera were also sent to Dr. P. Gemski at WRAIR for functional assays and for comparison with KLH-covalently coupled peptide vaccines. The data are summarized in TABLE VI. As shown, some of the peptides are highly immunogenic and elicit high titer of antibodies against the intact toxin. There effect on the toxin activity is under evaluation at WRAIR.

3.3. SECRETORY IMMUNITY.

3.3.1. Secretory Immunity in Rabbit Colostrum.

Ricin A peptides RA144 and lauryl-cysteine-RA144 were purified in large quantity to make vaccines for oral immunization of pregnant rabbits to test colostrum and lung lavage for secretory IgG and IgA. The protocol included SEB alone or coupled to beads, Ricin A alone or coupled to beads, RA144 alone or coupled to beads, or lauryl-cysteine or proteosomes. All the vaccines tested did not lead to high antibody titers in colostrum or lung lavage following oral immunization of rabbits.

3.3.2 Secretory immunity Respiratory and Intestinal Tracts

Murine system was developed to evaluate induction of IgA in the respiratory tract and protection against respiratory infection following intranasal immunization using peptide vaccines. Mice immunized intranasally with Ricin A or Ricin B peptides complexed to proteosomes induced iow levels of specific IgA and IgG in bronchial lavage fluids and sera, respectively. Cholera toxin B chain (CTB) enhanced immunogenicity when mixed with free Ricin A peptides or with Ricin B peptides complexed to proteosomes. Mice were immunized intranasally with Ricin A or Ricin B peptides complexed to proteosomes with a new protocol designed to induce higher levels of antibody (2 series on alternating days of immunizations, 3 weeks apart). Titers of specific IgA and IgG of 4-25 fold higher than those induced by previous protocols were found in bronchial lavage fluids and sera. As with the previous study, cholera toxin B chain (CTB) enhanced immunity when mixed with free Ricin A (but not Ricin B) peptides and, to a lesser extent, when mixed with peptides complexed to proteosomes. In another set of experiments mice were immunized intranasally with three Ricin A peptides that were recognized by the protective anti-Ricin A mAb (RA91-117, RA91-110 and RA96-110). Peptides that were hydrophobically complexed to proteosomes were found more potent in inducing anti-Ricin IgA and IgG in bronchial and intestinal lavage fluids and sera than those linked covalently to carrier protein, KLH. Priming intranasally was found superior to parenteral priming. Remarkably high anti-Ricin titers were obtained with RA96-110 complexed to proteosomes (serum titer >400 at o.d 1.0 for bronchial IgA and 200 for sera IgA in some animals).

Experiments with influenza peptides indicate that combining the two different peptides in two vaccines given together can enhance immunogenicity and protection. Experiments with shigella LPS indicate that proteosomes markedly enhance respiratory, intestinal and serum IgA and IgG responses to oral or nasal immunization with this antigen. An adjuvant effect of CTB was found in respiratory fluids following oral immunization and vice-versa. The adjuvant effect of Lipid A may be synergistic with proteosomes since LPS alone and detoxified LPS plus proteosomes were ineffective and since the titers were 4-10 fold higher than the peptide vaccine titers which lacked added LPS.

4. DISCUSSION

Protection against biologic toxins necessitates the development of both gastrointestinal and respiratory secretory immunity as well as systemic immunity. The research work described hereby was carried out in two major directions. The first defines efforts to identify neutralizing epitopes on the desired toxins mainly Ricin and SEB and the second concentrates on developing new methods to enhance the immunogenicity of peptides representing the neutralizing epitopes in order to make them suitable for use as vaccines for inducing secretory and systemic anti toxin antibodies.

The toxin Ricin, isolated from the seeds of the castor plant *Ricinus Communis*, is a ribosome inhibiting protein that, like other plant toxins (Abrin and Modeccin), consist of two polypeptide chains linked by a disulfide bond: the B chain is a lectin that binds to cell surface receptors containing terminal galactose residues and thereby facilitates entry of the toxic A chain into the cell (1). To understand this toxin, we attempted to design peptide immunogens that would elicit antibodies that recognize that portion of Ricin A responsible for its toxic enzymatic activity. Based upon analysis of conserved amino acids in a family of ribosome-inhibiting proteins (33) and on the 3-dimensional structural analysis as determined by x-ray crystallography at 2.8 A resolution, a prominent cleft visualized in the structure of Ricin A chain was suggested as the likely active site of the enzyme (29). Four amino acids deep in the cleft, Glu 177, Arg 180, Asn 180 and Trp 211 appear to participate in enzymatic activity and site directed mutagenesis of Ricin A indicates that Glu 177 is a key catalytic residue and Asn 209 is important for substrate binding (34).

Accordingly, we synthesized a series of eight overlapping peptides encompassing the 130 amino acids between RA 91 and RA 221 that delineate the cleft surrounding the proposed enzymatic site. An anti- Ricin A chain mAb that neutralized Ricin toxicity both *in vivo* and *in vitro* was used as a probe to identify

peptide sequences associated with neutralization. Of the eight initially selected peptides that spanned the entire cleft region, only RA 91-117 inhibited binding of the neutralizing mAb to Ricin A. By testing a series of nested peptides that were progressively shortened from either the amino or carboxyl termini, the minimal epitope recognized by this mAb was localized to the 10-mer, RA 97-106, defined by the sequence NQEDAEAITH. This peptide consists of a small helix interposed in a region of six ß stranded sheets. It is significant that according to the proposed structure of Ricin A, this sequence is found at the edge of the entrance to the cleft area. An important message of this analysis is that the epitope defined by the only known anti-Ricin A neutralizing mAb recognizes an area overlooking the entrance to the active site. In contrast, the site itself, located in the molecule's interior, is apparently not a preferred target for neutralizing antibodies. This understanding may assist attempts to define neutralizing epitopes of other toxins or enzymes.

The success in identifying the epitope recognized by the neutralizing mAb prompted the efforts to make a peptide vaccine that might induce neutralizing antibodies since the portion of Ricin A that should most likely be used in such vaccines was known. Hence, mice were immunized with vaccines containing each of the peptides recognized by the neutralizing mAb. In addition, vaccines containing peptides from adjacent segments encompassing the entire cleft area were also used in order to explore as many areas as possible in the vicinity of the enzymatic cleft. Both proteosome and KLH conjugated peptides were used with many of the peptides. Although most of these antisera (especially those elicited by the proteosome vaccines) recognized the intact protein, Ricin A, as well as the constitutive peptides with high titers, none of the polyclonal anti-peptide antisera neutralized Ricin either *in vitro* or *in vivo*.

In order to understand why antisera induced by peptides containing the epitope recognized by the neutralizing mAb could not neutralize the toxin, the fine specificity of the mAb was compared to that of the polyclonal antipeptide antisera. These results demonstrated that vaccines using peptides 91-110 or even 96-110 induced antibodies that preferentially recognized the carboxy terminal segment (101-110) of the immunizing peptide. In contrast, the mAb does not bind to this segment (101-110) and preferentially recognizes amino acids shifted towards the amino terminus of the peptide including 96-106 and 96-107, peptides recognized relatively poorly by the polyclonal antisera. These differences in the fine specificity provide one possible explanation for the lack of neutralizing by the polyclonal antipeptide antisera. Furthermore, when attempts were made to force the induction of antibodies that recognized this part of the peptide by immunizing with the shorter peptides 96-106 and 96-107, the antibodies induced recognized Ricin A very poorly even though their anti-peptide specificity was demonstrated. These antisera also did not recognize Ricin.

Another explanation for the lack of neutralization of the polyclonal anti-peptide antisera induced by peptides recognized by the mAb was furnished by

ELISA inhibition studies that compared the ability of soluble Ricin A protein to inhibit binding of either these polyclonal antisera or the mAb to solid phase Ricin A (as insoluble antigen fixed to the ELISA plate). This analysis demonstrated another difference between the neutralizing mAb and these polyclonal antisera in that very small amounts of soluble Ricin A inhibited the mAb's binding to solid phase Ricin A whereas even high concentrations of soluble Ricin A could not inhibit the binding of these antisera. These results show that the mAb recognizes a native conformational epitope expressed by Ricin A in solution whereas the antisera induced by the peptides recognized by the mAb do not. These data explain the lack of biological activity of the polyclonal antisera, since even when Ricin neutralization was tested *in vitro,* the assay allowed the antibodies to bind to Ricin in its native form in solution. The fact that the mAb recognized the peptides in solution suggests that the mAb influenced the conformation assumed by the soluble peptides. Indeed, it has been shown that antibodies may alter the exposed molecular surface of an antigen i.e. that in binding to antigens antibodies may assume the optimal complimentary conformation for binding by disruption of surface exposed salt bridges and hydrogen bonds. In contrast, in the absence of the mAb, when the peptides were used as immunogens, the dominant conformation of the peptides was sufficiently different from that of the native protein so as to generate the induction of antibodies that did not recognize soluble Ricin even though they did recognize the conformation assumed by Ricin A fixed to the plate.

These negative results, provide insight into understanding the basis for the inability of many peptide based vaccines to induce antibodies that are biologically effective against the native antigen (35-38).

The data strongly suggested to us that the neutralizing epitope on Ricin A chain is conformation-dependent and that the conformation assumed by the peptides used in the vaccines tested did not mimic its configuration in the native protein. Nevertheless, the fact that the neutralizing mAb did recognized both the peptide in solution and its original conformation on the native protein indicated that it is possible for the peptide to assume the desired conformation. We have postulated therefore that the effectiveness of synthetic peptide vaccines may be improved by using chemical means to stabilize its conformation to that assumed by the native protein. Towards that end, we were aided by the 3-dimensional modeling of the conformation of the native molecule as well as that of the proposed peptides used in potential vaccines. Computer analysis of the projected conformation of Ricin in the region recognized by the neutralizing anti Ricin A chain antibody suggested the use of RA90-110 as immunogen that by appropriate reduction and oxidation of two cysteines designed at both peptide termini, will undergo cyclization and be fixed as to mimic the natural conformation of the native protein. In parallel the same peptide was synthesized with extension of Ala-Ala-Ala inserted between the Cysteine and the lauric acid at the peptide carboxy terminus to provide the peptide with more flexibility necessary for equilibrium at the desired natural conformation. Indeed, antisera raised against the two conformation fixed peptides were found more reactive with native Ricin

A, as they were better inhibited by native soluble Ricin A. However, the antisera failed to neutralize Ricin toxicity *in vitro*. No neutralization was obtained also when the antisera were mixed with Ricin before injection to animals. Feasibility of such neutralization, in a narrow Ricin dose range, was proved under the same conditions when mAb to Ricin B was mixed with the toxin.

Neutralization was demonstrated only when animals immunized with the cyclic peptides were shown to be resistant to Ricin in the above narrow dose range described for neutralization upon mixture with the neutralizing mAb to Ricin B. It is suggested that the failure to abolish Ricin toxicity in passive immunization in the described trials was due to an insufficient amount of neutralizing antibodies (dictated to us by their limited availability) compared to the amounts found and induced in response to the toxin administration in immunized animals.

Since a possible encounter with biologic toxins may involve initial exposure via a mucosal surface, attempts to induce effective protection against them should rely on stimulation of the mucosal immune system in addition to the systemic response. In the case of viral pathogens it was shown that antiviral antibodies which are generated in respiratory mucosa play an essential role in protection against influenza infection (39). How[illegible], most nonliving non-replicating antigens are relatively ineffective of eliciting a mucosal response (40). An exception is the toxin produced by vibrio cholerae, which has been shown to be a potent mucosal immunogen by a number of investigators (41,42). The binding subunit of cholera toxin (CTB) was shown to be an effective carrier protein for mucosal immunity to covalently linked proteins (43-45). Since proteosomes were shown highly effective in enhancing systemic immune response against peptides even without added adjuvants, we suggested them as possible candidates as carriers for peptides for the induction of protective immunity in mucosaltissues. The development of secretory antibodies (of all classes) in lungs and gastrointestinal secretions as well as serum antibodies (especially IgA) was measured . CTB and LPS were examined for their synergistic roles as associated oral adjuvants. The results obtained emphasized the importance of the immunization protocol. Whereas one intranasal immunization with proteosomes linked peptides induced low levels of specific IgA and IgG in bronchial lavage fluids, when a second protocol including 2 series of injections 3 weeks apart of 2 daily immunization, 4-25 fold higher titers were obtained for both IgA and IgG. In an experiment designed to measure induction of anti Ricin IgA and IgG using 3 Ricin A peptides that were recognized by the protective anti Ricin A mAb, linkage to proteosomes was found more effective in inducing secretory immunity than linkage to KLH. Priming intranasaly was found superior to parenteral priming. CTB was found to enhance immunity when mixed with free peptide and to a lesser extent when mixed with proteosomes vaccine. Experiments with Shigella LPS indicate that proteosomes markedly enhance respiratory, intestinal and serum IgA and IgG responses to oral or nasal immunization with this antigen.

The practical merit of intranasal vaccination is obvious. Intranasal inoculation can be performed easily and painlessly. It is technically possibly to inoculate relatively large amounts of immunogen. Anti peptides antibodies in intestinal secretion were detected after intranasal immunization indicating that antigenic stimulation of bronchus associated lymphoid tissue (BALT) as well as gut associated lymphoid tissue (GALT) is able to induce specific IgA antibodies in intestinal secretions probably accounting for the migration of lymphocytes sensitized in BALT to GALT.

In conclusion, the results obtained under this study demonstrate the ability of synthetic peptide based vaccines to elicit neutralizing antibodies against a lethal toxin such as Ricin. Furthermore, we demonstrate that by the employment of proteosomes, which serve as both a carrier and an adjuvant, it is possible to replace the hazardous form of toxoid employed in conventional vaccines formulae. Finally, such peptide-proteosome conjugates are effective in inducing mucosal immunity as a response to oral or intranasal vaccination, which could be of high practical merit.

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ACRONYMS AND SYMBOLS.

a) The following standard single letter designations for amino acids have been used when giving peptide sequences in this proposal.

A=ala, C=cys, D=asp, E=glu, F=phe, G=gly, H=his, I=ile, K=lys, L=leu, M=met, N=asn, P=pro, Q=gln, R=arg, S=ser, T=thr, V=val, T=trp, Y=tyr.

b) **Other acronyms used:**

BSA	=	bovine serum albumin
KLH	=	keyhole lympet hemocyanin
SEB	=	staphylococcal enterotoxin B
NTP2	=	scorpion neurotoxin P
CTB	=	cholera toxin B subunit

LEGENDS TO FIGURES

FIGURE 1. Solution phase peptide binding to monoclonal antibody neutralizing Ricin toxicity. Binding of constant amounts of antibody (2.10^{-10}M) to Ricin A as antigen on the plate was measured in the presence of increasing concentrations of the different peptides.

FIGURE 2. Solution phase binding of RA 91-116 derivatives to monoclonal antibody neutralizing Ricin toxicity. Binding of constant amount of antibody (2.10^{-10}M) to Ricin A as antigen in the presence of the peptides. Results at 16.6 and 50ug/ml peptide in the medium are listed.

FIGURE 3. Peptide binding to antibodies directed against selected Ricin A peptides and to an anti-Ricin A monoclonal antibody.

FIGURE 4. Solution phase binding of monoclonal antibody to Ricin A and antisera raised to the different peptides to Ricin A. Binding of constant amounts of antibody (2.10^{-10}M), or 1:800 dilution of antisera, to plate bound Ricin A was measured in the presence of increasing concentrations of scluble Ricin A. Results are presented as percentage of binding inhibition and represent means of triplicates.

FIGURE 5. Effect of monoclonal antibody to Ricin A and different antisera to Ricin peptides on Ricin toxicity to NSO cells. Different amounts of Ricin were preincubated overnight at 4^0C with either monoclonal antibody to Ricin A (A) or antisera raised to the different peptides (B). One hundred thousand NSO cells were then added and incubation was carried overnight at 37^0C.MTT reaction was developed as described (28). Results are presented as % of living cells.

FIGURE 6. Solution phase binding of antisera to cyclic RA90-110 AND RA-Ala-90-110 to Ricin A. Binding of 1:200 dilution of antisera to plate bound Ricin A was measured in the presence of increasing concentrations of soluble Ricin A. Results are presented as percentage of binding inhibition and represent means of triplicates.

TABLE 1a

IMMUNOGENICITY OF RICIN A PEPTIDE VACCINES USING PROTEOSOME-LIPOPEPTIDES (IN NS) OR KLH-PEPTIDES (IN CFA): TERTIARY MURINE IGG RESPONSES AS MEASURED BY ELISA

Area	Carrier/ Adjuvan	Peptide Location (C=added Cysteine)	Reciprocal Serum Titer (O.D. >0.5) Against	
			Ricin A Protein	Homologous Peptide
1	Proteosome	C144-170	3,200	1,600
	KLH/CFA	C144-170	6,400	1,600
2	Proteosome	C161-186	3,200	3,200
	Proteosome	C161-186	3,200	3,200
	KLH/CFA	C161-186	50	6,400
3	Proteosome	C171-198	200	800
	Proteosome	C171-196	6,400	12,800
	KLH/CFA	C171-198	3,200	6,400
4	Proteosome	C190-215	51,200	6,400
	KLH/CFA	C190-215	1,600	800
5	Proteosome	C199-221	<50	400
	KLH/CFA	C199-221	<50	50
6	Proteosome	C 91-117	1,600	3,200
7	Proteosome	C107-132	1,600	3,200
8	Proteosome	C122-148	<50	400
5a	Proteosome	C205-213	50	200

TABLE 1b

IMMUNOGENICITY OF RICIN A PEPTIDE VACCINES USING PROTEOSOME-LIPOPETIDES (IN NS) OR KLH-PEPTIDES (IN CFA): TERTIARY-MURINE IGG RESPONSES AS MEASURED BY ELISA

Area	Carrier/ Adjuvant	Peptide Location (C=added Cysteine)	Ricin A Protein	Homologous Peptide
1	Proteosome	C96 - 106	100	1,600
	KLH/CFA	C96 - 106	50	400
2	Proteosome	C96 - 107	200	1,600
	KLH/CFA	C96 - 197	50	100
3	Proteosome	C96 - 108	100	100
	KLH/CFA	C96 - 108	50	50
4	Proteosome	C96 - 109	100	300
	KLH/CFA	C96 - 109	50	50
5	Proteosome	C97 - 110	100	100
6	Proteosome	C98 - 100	100	400
7	Proteosome	C99 - 110	100	800
8	Proteosome	C100-110	50	200

TABLE II

EFFECT OF A MONOCLONAL ANTIBODY AGAINST RICIN A[a]

Ricin/Mouse	Antibody	Day of Death	Molar Ratio (Mab:Ricin)
0.125	mAb to ricin A chain (30mg)	-	10:1
0.250	mAb to ricin A chain (30mg)	-	5:1
0.500	mAb to ricin A chain (30mg)	4,4,4,5	2.5:1
0.125	mAb to EGF R (30mg)	15,-,-,-	
0.250	mAb to EGF R (30mg)	3,4,7,9	
0.50	mAb to EGF R (30mg)	2,2,3,3	
0.125	PBS	-	
0.250	PBS	5,6,15,19	
0.500	PBS	2,4,4,-	
0.33	Proteosomes RA91-116[b]	2,2,3	
0.33	KLH RA91-116[b]	2,2,2	
0.33		2,3,3	

a Ricin at different doses was incubated as described in Materials and Methods, with monocolonal antibodies and different antisera before injection to animals

b Antisera were precipitated with 50% $(NH_4)\ SO_4$ And 120ml of equivelant serum antibody content (See table 1 for titer) were injected.

TABLE III

NEUTRALIZATION OF RICIN TOXICITY BY INCUBATION WITH DIFFERENT ANTISERA BEFORE ADMINISTRATION TO MICE

Antisera for Neutralization[1]	Ricin (µg/mouse)	Day of Death[2]	Survival at Termination
PBS	0.5	5 5 7 7	1/5
	0.25	7 26 - - -	3/5
	0.125	- - - - -	5/5
PRAL RA90-110	0.5	5 7 7 - -	2/5
	0.25	3 5 5 7 12 -	1/6
	0.125	- - - - -	5/5
PRAL RA-Ala90-110	0.5	5 5 7 - -	2/5
	0.25	7 7 7 7 7	0/5
	0.125	- - - - -	5/5
MAB to Ricin B	0.5	777	2/5
(64.20)	0.25	-- - - -	5/5
	0.125	-- - - - -	5/5

[1]Ricin at the indicated dose was mixed with 20ml of sera from animals immunized with the indicated vaccine (3 i.p. injections of 50mg peptide complexed to proteosomes). Titers of sera were 400 and 800 for PRAL RA90-100 and PRAL Ala90-110 respectively when measured against Ricin A in ELISA (O.D.>0.5). For the Mab 50mg per mouse were mixed with the indicated dose of Ricin. Injection was given i.p. after 1 hr incubation at 4^0C in the presence of carrier BSA.

[2]Day after Ricin administration

TABLE IV

RESISTANCE OF IMMUNIZED MICE TO RICIN TOXICITY

Vaccination[1]	Ricin (ug/mouse)	Day of Death[2]	Survival
PBS	0.5	3 5 5 7 -	1/5
	0.25	7 7 7 12 -	1/5
	0.125	- - - - -	5/5
PRAL RA90-110	0.5	3 5 7 7 -	1/5
	0.25	3 - - - -	4/5
	0.125	- - - - -	5/5
PRAL RA-Ala90-110	0.5	5 5 5 5 5	0/5
	0.25	5 5 - - - -	4/6
	0.125	- - - - - -	6/6

1 Animals were given 3 i.p. injection of 50mg peptide complexed to proteosomes at days 1, 22, 38. At day 60 Ricin at the indicated dose was administered i.p and animals mortality was followed.

2 Day after Ricin administration

TABLE Va

IMMUNOGENICITY OF PROTEOSOME-LIPOPETIDE (IN NS) OR KLH-PEPTIDE (IN CFA) VACCINES FROM THE B CHAIN OF RICIN: TERTIARY PEPTIDES AS MEASURED BY ELISA

Ricin Area	Carrier/ Adjuvant	Source of Peptide	Peptide Location (C=added Cysteine)	Reciprocal Serum Titer (O.D. > 0.5) Against:	
				Homologous Protein	Homologous Peptide
9	Proteosome	Ricin B	C230-257	100	3,200
10	Proteosome	Ricin B	C244-262	200	1,600
10	Proteosome	Ricin B	C244-262	200	50
9a	Proteosome	Ricin B	C232-239	<50	400
10a	Proteosome	Ricin B	C247-256	50	400
11	Proteosome	Ricin B	C20-47	50	6,400
11a	Proteosome	Ricin B	C20-28	50	1,600
11b	Proteosome	Ricin B	C33-47	< 50	800

TABLE V b

IMMUNOGENICITY OF PROTEOSOME-LIPEPTIDE VACCINES FROM THE A OR B CHAINS OF RICIN: SECONDARY AND TERTIARY MURINE IGG RESPONSES AGAINST HOMOLOGOUS RICIN PROTEINS OR PEPTIDES AS MEASURED BY ELISA

Ricin	Proteosomes: Std=standard I=Israel (new) R=Rockefeller MgC=meningo GC=gonococci X=genitically lacking Class 3	Source of Peptide	Peptide Location (C=added)	Reciprocal Serum Titer O.D. < 0.5 Against:			
				Homologous Protein		Homologous Peptide	
				2°	3°	2°	3°
5a	Std.MgC	Ricin A	C205-213	<50	50	200	200
9	Std. MgC	Ricin B	C230-257	50	50	3,200	25,600
9	MgC-I	Ricin B	C230-257	50	1,600	800	12,800
9	MgC-R	Ricin B	C230-257	<50	200	50	3,200
9	GC-Std-R	Ricin B	C230-257	100	200	800	3,200
9	GC-X-R	Ricin B	C230-257	400	400	800	1,600
9a	Std.MgC	Ricin B	C232-239	<50	<50	200	400
10a	Std.MgC	Ricin B	C247-256	<50	50	400	400
11	Std.MgC	Ricin B	C20-47	<50	50	800	6,400
11a	Std.MgC	Ricin B	C20-47	<50	50	100	1,600
11b	Std.MgC	Ricin B	C33-47	>50	>50	100	800

TABLE VI

IMMUNOGENICITY OF PROTEOSOME-LIPOPEPTIDE VACCINES USING SEB PEPTIDES: TERTIARY AND QUATERNARY MURINE IgG RESPONSES AS MEASURED IN AN ELISA

No.	Peptide Location	Reciprocal Serum Titre (O.D. >0.5) Against: SEP Protein		Homologous Peptide
		tertiary	quaternary	tertiary
1	1-30	3,200	6,400	1,600
2	21-50	800	12,800	800
3	41-70	3,200	12,800	102,000
4	61-92	25,000	102,400	204,000
6	93-112	<50	<50	102,000
7	101-130	<50	<50	200
8	113-144	12,800	25,600	102,000
9	130-160	6,400	12,800	204,000
10	151-180	6,400	800	800
11	171-200	800	800	800
12	191-220	400	1,600	400
13	210-239	400	400	50

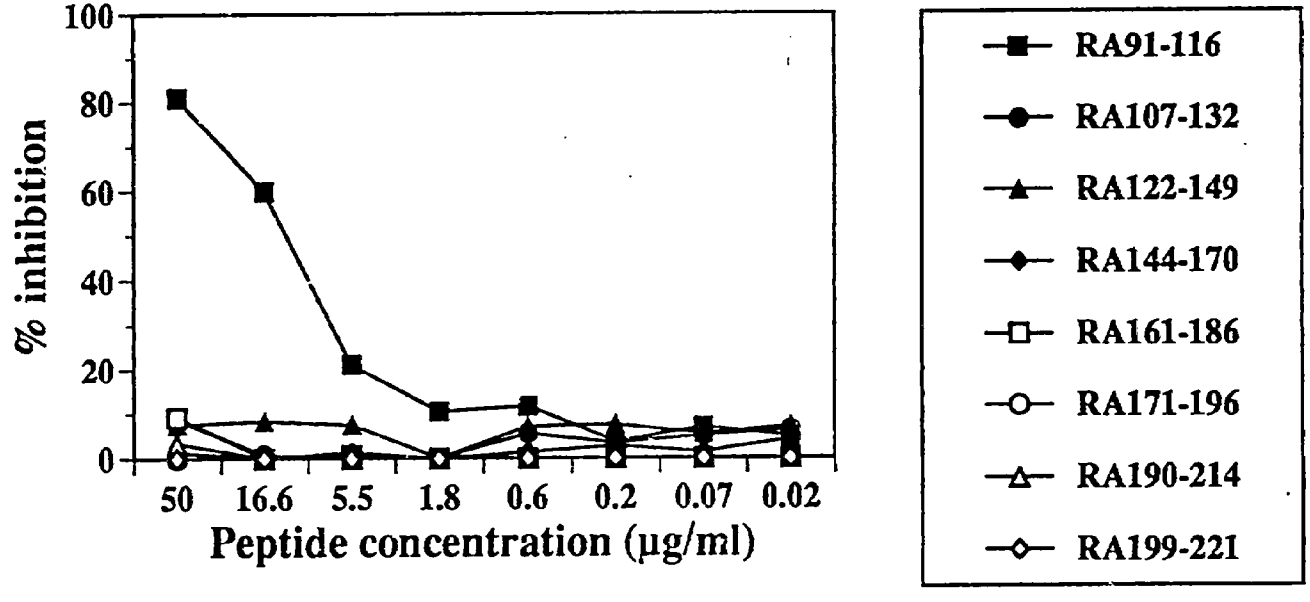
100
80
60
40
20
0
% inhibition
50
16.6
5.5
1.8
0.6
0.2
0.07
0.02
Peptide concentration (µg/ml)
RA91-116
RA107-132
RA122-149
RA144-170
RA161-186
RA171-196
RA190-214
RA199-221

Fig 1

	Inhibition (%)	
	50 µg	16.5 µg
RA91 -116	75	45
RA91 -110	66	35
RA91 -100	21	22
RA96 -110	81	58
RA97 -110	78	51
RA98 -110	37	18
RA99 -110	29	26
RA100-110	29	23
RA101-110	24	25
RA96 -109	78	45
RA96 -108	91	77
RA96 -107	87	61
RA96 -106	75	43
RA96 -105	24	25

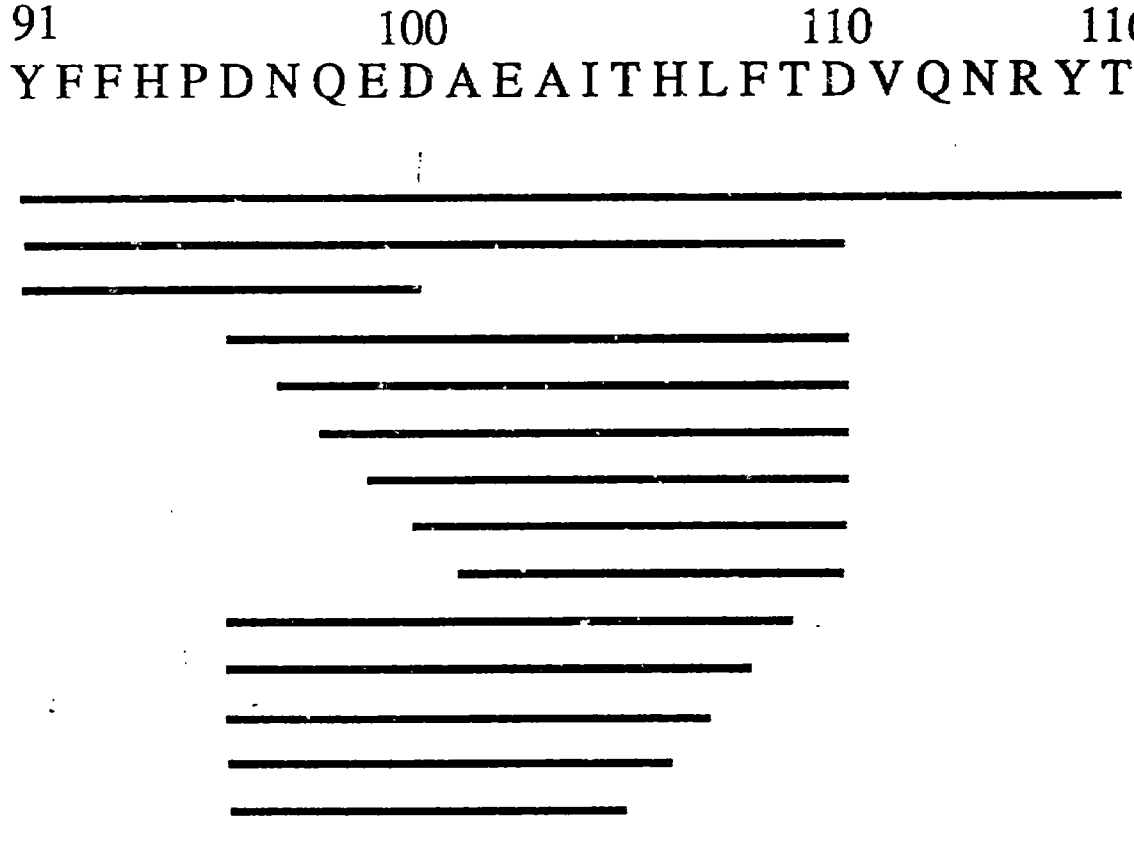

Fig 2

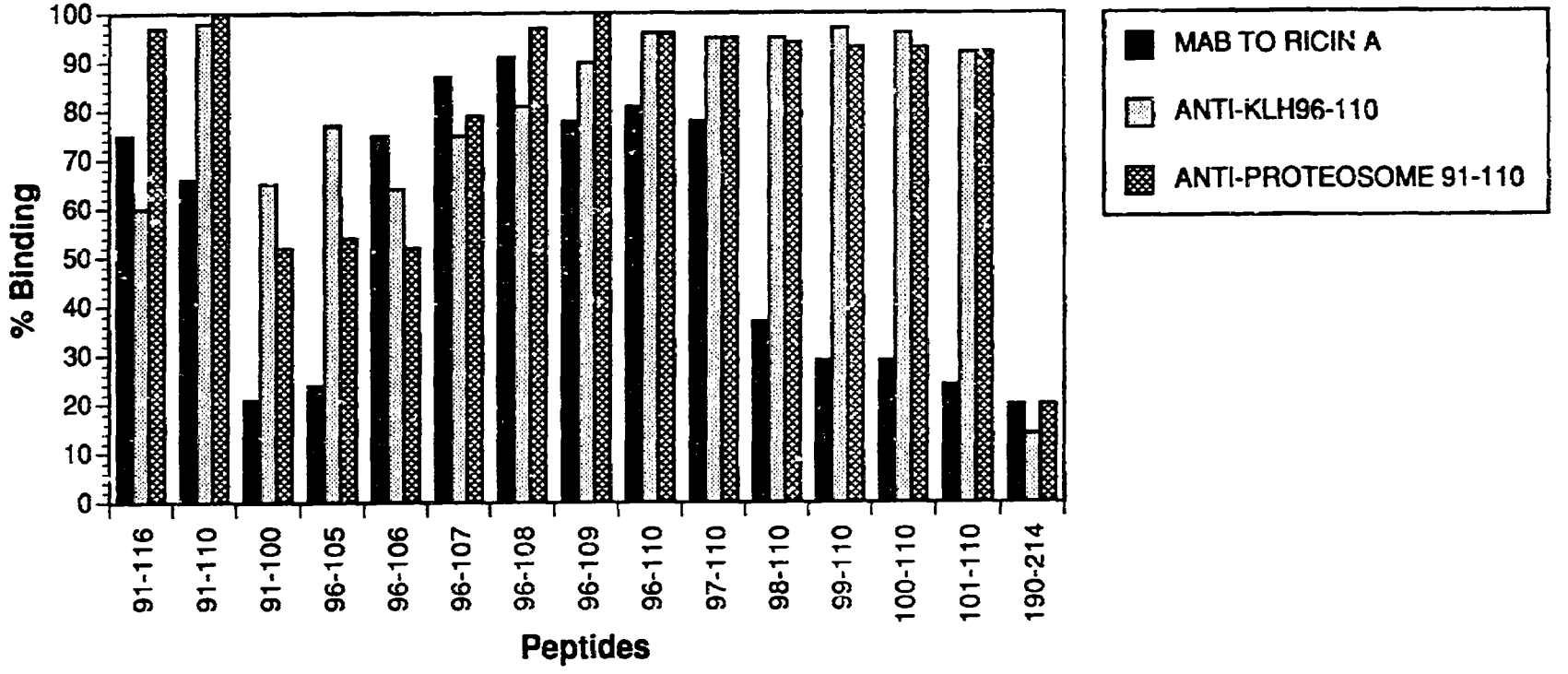
100
90
80
70
60
50
40
30
20
10
0
% Binding
91-116
91-110
91-100
96-105
96-106
96-107
96-108
96-109
96-110
97-110
98-110
99-110
100-110
101-110
190-214
Peptides
MAB TO RICIN A
ANTI-KLH96-110
ANTI-PROTEOSOME 91-110

Fig 3

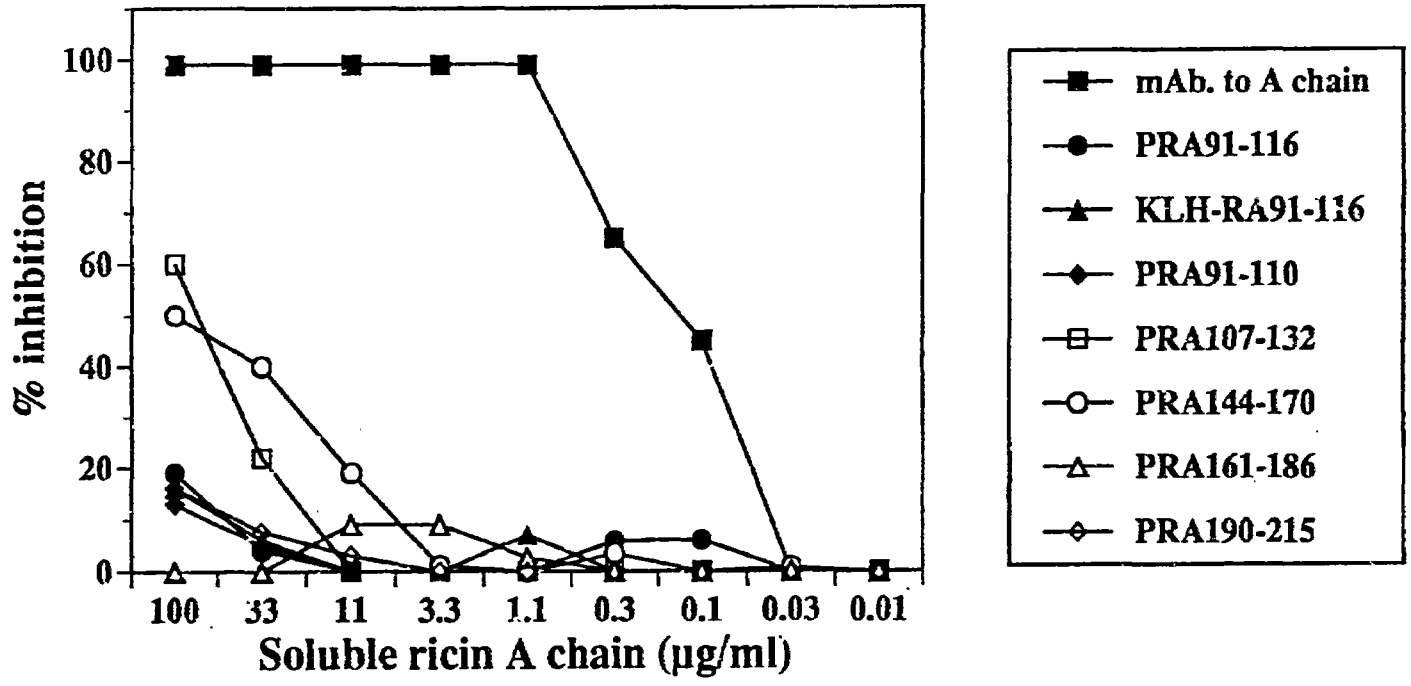
100
80
60
40
20
0
% inhibition
100
33
11
3.3
1.1
0.3
0.1
0.03
0.01
Soluble ricin A chain (µg/ml)
mAb. to A chain
PRA91-116
KLH-RA91-116
PRA91-110
PRA107-132
PRA144-170
PRA161-186
PRA190-215

Fig 4

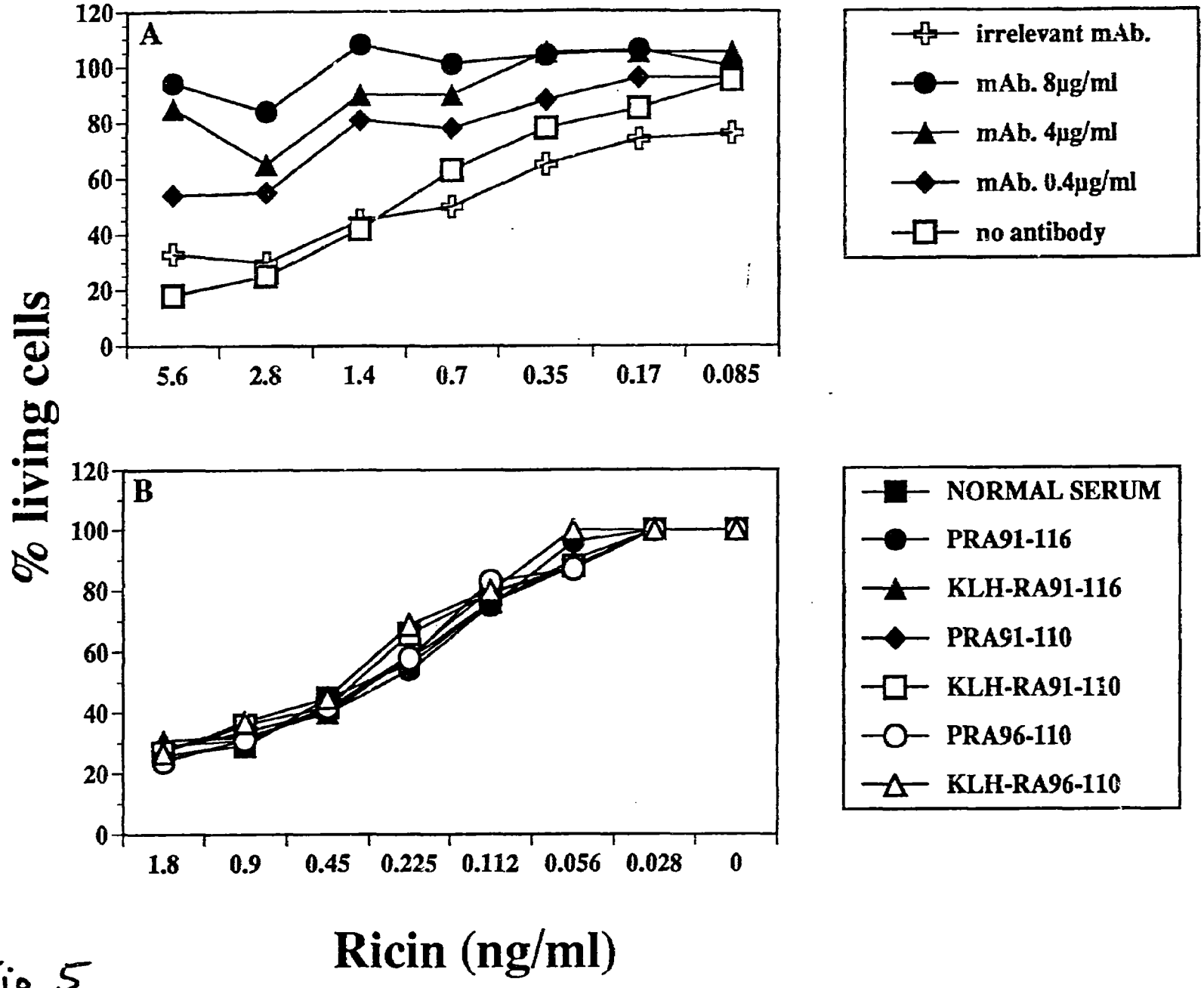
A
B
120
100
80
60
40
20
0
5.6
2.8
1.4
0.7
0.35
0.17
0.085
1.8
0.9
0.45
0.225
0.112
0.056
0.028
0
% living cells
Ricin (ng/ml)
irrelevant mAb.
mAb. 8µg/ml
mAb. 4µg/ml
mAb. 0.4µg/ml
no antibody
NORMAL SERUM
PRA91-116
KLH-RA91-116
PRA91-110
KLH-RA91-110
PRA96-110
KLH-RA96-110

Fig 5

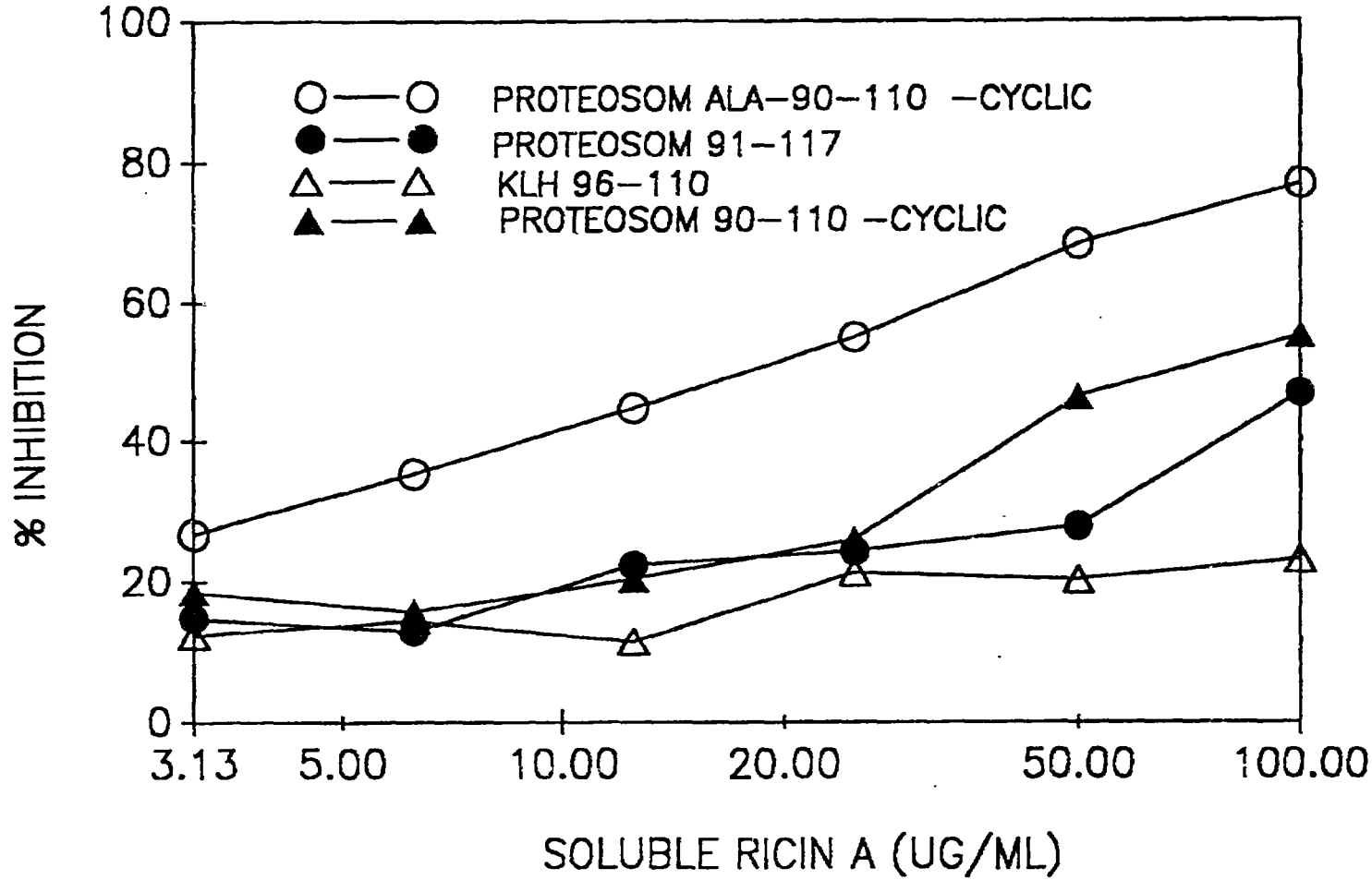
100
80
60
40
20
0
% INHIBITION
PROTEOSOM ALA–90–110 –CYCLIC
PROTEOSOM 91–117
KLH 96–110
PROTEOSOM 90–110 –CYCLIC
3.13
5.00
10.00
20.00
50.00
100.00
SOLUBLE RICIN A (UG/ML)

Fig 6